Germline stem cell number in the *Drosophila* ovary is regulated by redundant mechanisms that control Dpp signaling

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Accepted 14 January 2004

Development 131, 1881-1890
Published by The Company of Biologists 2004
doi:10.1242/dev.01076

Summary

The available experimental data support the hypothesis that the cap cells (CpCs) at the anterior tip of the germarium form an environmental niche for germline stem cells (GSCs) of the *Drosophila* ovary. Each GSC undergoes an asymmetric self-renewal division that gives rise to both a GSC, which remains associated with the CpCs, and a more posterior located cystoblast (CB). The CB upregulates expression of the novel gene, *bag of marbles* (*bam*), which is necessary for germline differentiation. Decapentaplegic (Dpp), a BMP2/4 homologue, has been postulated to act as a highly localized niche signal that maintains a GSC fate solely by repressing *bam* transcription. Here, we further examine the role of Dpp in GSC maintenance. In contrast to the above model, we find that an enhancer trap inserted near the Dpp target gene, *Daughters against Dpp* (*Dad*), is expressed in additional somatic cells within the germarium, suggesting that Dpp protein may be distributed throughout the anterior germarium. However, *Dad-lacZ* expression within the germline is present only in GSCs and to a lower level in CBs, suggesting there are mechanisms that actively restrict Dpp signaling in germ cells. We demonstrate that one function of Bam is to block Dpp signaling downstream of Dpp receptor activation, thus establishing the existence of a negative feedback loop between the action of the two genes. Moreover, in females doubly mutant for *bam* and the ubiquitin protein ligase *Smurf*, the number of germ cells responsive to Dpp is greatly increased relative to the number observed in either single mutant. These data indicate that there are multiple, genetically redundant mechanisms that act within the germline to downregulate Dpp signaling in the Cb and its descendants, and raise the possibility that a Cb and its descendants must become refractory to Dpp signaling in order for germline differentiation to occur.

Key words: Decapentaplegic, Dpp, Stem cell, GSC, Bam, Smurf, *Drosophila*, Germline

Introduction

A stem cell is an undifferentiated cell that can undergo an asymmetric self-renewal division to produce one descendant like itself and one descendant whose progeny ultimately differentiate into a defined cell type (Fuchs and Segre, 2000). One hypothesis to explain this pattern of asymmetric cell division is that the microenvironment of the stem cell, called an 'environmental niche', permits the stem cell to remain in an undifferentiated state, while removal of one of its daughters from the niche results in the onset of differentiation (Spradling et al., 2001; Watt and Hogan, 2000).

The anatomy of the germarium of the *Drosophila* ovariole is consistent with the existence of a niche that maintains the GSCs (Fig. 1A). Two or three GSCs lie at the anterior tip of the germarium in contact with somatic CpCs and in close apposition to somatic terminal filament (TF) cells. The plane of GSC division is perpendicular to the anteroposterior axis of the germarium such that the daughter cell that stays in contact with the CpCs remains a GSC, while the more posterior daughter cell becomes a cystoblast (CB). Each CB divides four times with incomplete cytokinesis, resulting in a germline cyst containing 16 interconnected cells, one of which will become the oocyte.

A variety of experiments strongly suggest that somatic cells that surround the GSCs form an environmental niche necessary for GSC survival and renewal. First, if a GSC is lost due to mutation or age, the niche provides an environment for the replenishment of the GSC from a presumptive CB cell (Xie and Spradling, 2000). Second, cell adhesion between the CpCs and GSCs is necessary for GSC maintenance (Song et al., 2002). Third, the niche is likely to send intercellular signals to the GSCs (Xie and Spradling, 1998; King et al., 2001). The best characterized of the niche signals is the BMP 2/4 homologue Dpp (Xie and Spradling, 1998; Xie and Spradling, 2000). *dpp* is expressed in the CpCs and the more posterior inner sheath cells (ISCs) (Xie and Spradling, 2000; Zhu and Xie, 2003). Dpp signaling within the GSCs is necessary for GSC maintenance, as clones of GSCs homozygous mutant for Dpp signaling components such as the Smad family members, *Mad* or *Medea*, or the type I BMP receptors *thickveins* (*tkv*) and *saxophone* (*sax*), are not maintained over time (Xie and Spradling, 1998). Moreover, ectopic Dpp expression within all somatic cells of the germarium results in the overproliferation of germ cells with characteristics similar to GSCs (Xie and Spradling, 1998).

Conversely, there is at least one gene, *bag of marbles* (*bam*),

whose activity in CBs is necessary to promote germline differentiation. Females that lack *bam* activity have ovarioles that contain an overproliferation of cells with GSC or CB-like morphology (McKearin and Ohlstein, 1995). The *bam* gene is repressed in GSCs under control of Dpp signaling (Chen and McKearin, 2003a), but is expressed in CBs (McKearin and Spradling, 1990; Chen and McKearin, 2003b). Ectopic expression of *bam* in GSCs results in their elimination (Ohlstein and McKearin, 1997). Bam function requires the activity of *benign gonadal cell neoplasm* (*bgcn*), whose predicted protein product shares several motifs with the DexH family of RNA helicases (Lavoie et al., 1999; Ohlstein et al., 2000), suggesting that Bam and Bgcn act together to promote GSC differentiation by post-transcriptional regulation of gene activity (Ohlstein et al., 2000). These data suggest a model for control of the asymmetric GSC division in which the anterior GSC daughter has a high level of Dpp signaling and maintains a GSC fate by repressing *bam* transcription, while the posterior GSC daughter has a lower level of Dpp signaling, thereby allowing *bam* expression, which triggers a program leading to CB differentiation.

In this paper, we examine the mechanisms underlying the temporal and spatial control of Dpp signaling within the developing germline. We find that, although Dpp signaling in the somatic cells is not limited to the niche, the expression of the Dpp target gene *Dad-lacZ* in the germline is confined to GSCs and CBs. We demonstrate that one, but not the only, function of Bam is to downregulate Dpp signaling downstream of Dpp receptor activation, and that action of the ubiquitin protein ligase Smurf (Lack – FlyBase) is functionally redundant with that of Bam in downregulation of Dpp signaling. These data provide potential insight into the mechanisms underlying the stable switch in developmental states that occurs during GSC differentiation.

Materials and methods

Stocks

Wild-type and mutant stocks were maintained on standard yeast-agar-cornmeal medium and all stocks were grown at 25°C. All alleles, aberrations and transgenes are described in FlyBase or the following references: sax^{B18} (S. Podos, Y.-C. Wang and E.L.F., unpublished) and *P{vas-egfp::vas}* (Sano et al., 2002). To create *P{UAS.p-TkvAct}*, flies were transformed with a P-element plasmid containing DNA from the coding region of a constitutively-active form of the Thickveins receptor (TKV Q253→D) (Neul and Ferguson, 1998) inserted at *Not*I and *Xba*I sites into the UASp plasmid (Rørth, 1998).

Immunocytochemistry, fluorescence and confocal microscopy

Ovaries were dissected in EBR buffer and stained as described (Lin et al., 1994). The following primary antibodies were used: monoclonal anti-Orb antibody (1:10) (Lantz et al., 1994); polyclonal anti-*lacZ* antibody (1:500, Cappel); monoclonal anti-Hts antibody 1B1 (1:2.5) (Zaccai and Lipshitz, 1996); monoclonal anti-Myc antibody 1-9E10 (1:100, Santa Cruz); polyclonal anti-alpha spectrin antibody (1:400) (Byers et al., 1987); polyclonal anti-Vasa antibody (1:1000) (Liang et al., 1994); rat polyclonal anti-Tkv antibody (1:5) (Teleman and Cohen, 2000); monoclonal anti-BamC antibody used as described by McKearin and Ohlstein (McKearin and Ohlstein, 1995); and polyclonal anti-GFP (1:500, Abcam). The following secondary antibodies were used: anti-rabbit, anti-mouse and anti-rat Alexa Fluor 488 (1:1000, Molecular Probes); and anti-mouse and anti-rabbit Cy3 (1:1000, Jackson Immunoresearch). DNA was visualized either by YOPRO1 (1 μM, Molecular Probes) or by DAPI (0.3 μM, Molecular Probes) staining. Mounting of samples was carried out in a 70%Tris-glycerol mixture pH 7.6 containing 2% DABCO (Sigma). Fluorescent images were captured with a Zeiss Axiocam mounted on a Zeiss Axioplan microscope equipped with a 20× Plan-Apo 1.4 NA objective. Digital images of serial optical sections were collected with a BioRad 1024 Zeiss confocal microscope using either 25× or 63× objectives. Images were merged using the LSM50 software and further processed using Adobe Photoshop 5.0.

Determination of niche size in wild-type and mutant females

The number of *Dad-lacZ*-expressing germ cells was determined in multiple ovarioles from females of each of the following genotypes (separated by commas): *P{lacZ}*Dad^{P1883}*/TM3*, *P{lacZ}*Dad^{P1883} bam^{D86}/bam^{D86}, *Tp(2;2)DTD48/CyO;* *P{lacZ}*Dad^{P1883}*/TM2*, sax^{B18}*/CyO;* *P{lacZ}*Dad^{P1883}*/TM2*, sax^{B18} *Tp(2;2)DTD48/CyO*; *P{lacZ}*Dad^{P1883}*/TM2*, sax^{B18} *Tp(2;2)DTD48/CyO;* bam^{D86}/bam^{D86} *P{lacZ}*Dad^{P1883}, $Smurf^{15C}$*;* bam^{D86} *P{lacZ}*Dad^{P1883}*/TM2* and $Smurf^{15C}$*;* bam^{D86}/bam^{D86} *P{lacZ}*Dad^{P1883}. For each ovariole from single mutant females, the number of cells expressing *lacZ* was counted using confocal microscopy. The statistical analyses of the number of GSCs in wild-type and single mutant ovarioles were performed using the GraphPad Prism program. We note that the survival to adulthood of double mutant flies of genotypes $Smurf^{15C}$*;* bam^{D86}/bam^{D86} *P{lacZ}*Dad^{P1883} and sax^{B18} *Tp(2;2)DTD48/CyO;* bam^{D86}/bam^{D86} *P{lacZ}*Dad^{P1883} was much lower than expected.

Phenotype of tumorous ovaries after Bam expression

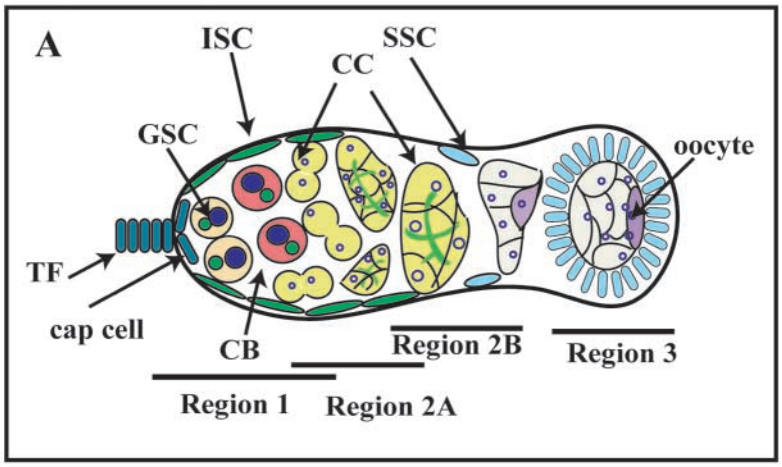
Flies of genotypes *P{hs-bam.O}; P{UAS.p-TkvAct)/TM3,Sb* and *P{Gal4::VP16-nos.UTR}* were mated at 25°C and transferred every day to new bottles. In experimental but not control crosses, on the seventh and following days after egg deposition, the F1 larvae/pupae were subject to two 1-hour 37°C heat shocks, separated by 1 hour at room temperature. After eclosion, experimental non-Sb F1 females were collected and kept in vials with fresh yeast at 25°C and heat-shocked daily using the same protocol. Dissected ovaries of F1 non-Sb control and experimental females of the same age were examined with Nomarski or confocal optics. Some experimental females were mated with wild-type males in small egg-laying cups for 3 days to examine the follicular morphology of the eggs. Application of the identical heat shock protocol caused females of genotype *P{hs-bam.O}* to lose all germ cells within their germaria.

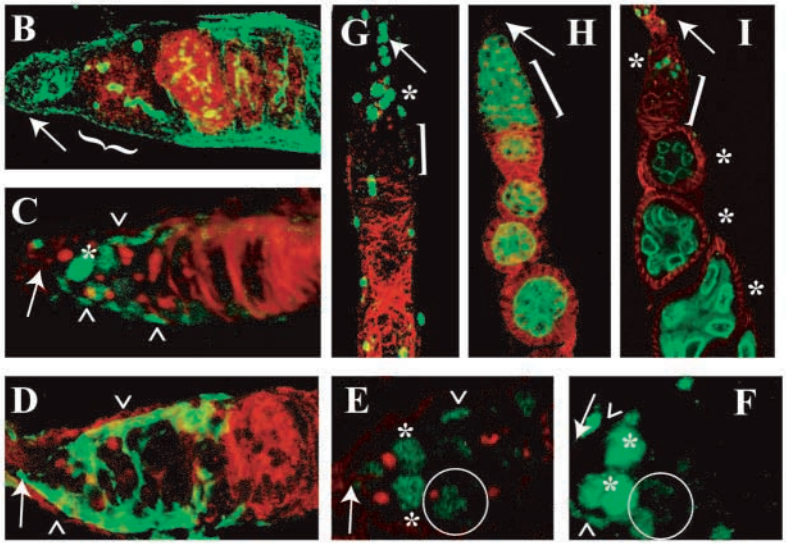
Nos and *Dad-lacZ* detection in tumorous ovaries after Bam expression

Flies of genotype *P{nos-myc.V}/CyO;* *P{UAS.p-TkvAct}* *P{lacZ}*Dad^{P1883}*/TM3,Sb* were mated with flies of genotype *P{hs-bam.O}*; *P{GAL4::VP16-nos.UTR}/TM3,Sb*. In experimental but not control crosses, F1 larvae/pupae were heat shocked as described above, beginning on day 9-10 of development and continuing for 1 or 2 days after eclosion. Ovaries from control and experimental non-Sb, non-Cy F1 females of the same age were stained at the same time, and image acquisition of control and experimental samples was performed at the same session. Specifically, control tumors were scanned first and the same acquisition parameters were used to scan the experimental samples. The images of control and experimental ovaries were subsequently processed identically. Each set of experiments was repeated at least three times.

Expression of *lacZ* in bam^{D86} mutant ovaries

Flies of genotypes *P{hs-bam.O}*; bam^{D86}*/ TM3,Sb* and *P{UAS-lacZ.p};* bam^{D86} *P{GAL4::VP16-nos.UTR}/TM3,Sb* were crossed. For the experimental but not control cross, F1 progeny were subject to heat shock using the protocol described above. Ovaries from F1 non-Sb experimental and control females were dissected 1-2 days after eclosion.





J	Stage	Spec	Fus	Bam	Dad	Nos	Orb
R1	GSC	+	-	-	+	+	-
	CB	+	-	+	+	+	-
	2-8 cc	-	+	+	-	-	-
R2A	16 cc	-	+	++	-	++	+
R2B	16 cc	-	+/-	-	-	+/-	+

Fig. 1. Identification of cell types within the germarium. (A) A representation of the germarium. The germline stem cells (GSCs) reside at the anterior tip of the germarium and divide asymmetrically such that one daughter becomes a cystoblast (CB) while the other daughter remains a GSC. GSCs are associated with terminal filament (TF) and cap cells (both somatic cells, dark blue). All GSCs and CBs contain spectrosomes (green dot) and express *Dad-lacZ* (blue dot). The CBs undergo four rounds of mitotic division with incomplete cytokinesis to produce 2, 4 and 8 germline cell cysts (CC) in Region 1 and 16 CC in Region 2A. All cyst cells are interconnected by a fusome (green lines). Germ cells are associated with inner sheath cells (ISCs) in Regions 1 and 2A of the germarium. In Region 2B, one cell of the 16 CC becomes specified as an oocyte (purple), and cysts become enveloped by follicle cells (light blue), derived from somatic stem cells (SSC). In Region 3, a stage 1 egg chamber buds off from the germarium. (B-I) Confocal projections of morphologically wild-type germaria or ovarioles. Arrow indicates position of terminal filament. (B) Germarium of female carrying a Nos-Myc transgene, showing low expression in GSCs and CBs (bracket), absence from two- to eight-cell cysts, and high expression in 16-cell cysts (anti-spectrin, green; anti-Myc, red). Germarium (C,E,F) and whole ovariole (G) of females of genotype *P{lacZ}Dad^{P1883}/ TM3,Sb*. (C,E,G) Anti-*lacZ* (green), anti-Hts (red). Bracket in G indicates region 2 of the germarium.(F) Anti-*lacZ* (green). *Dad-lacZ* expression in the germline is present at high levels in GSCs (asterisk) and at lower levels in CBs (circles in E and F), but expression of *Dad-lacZ* is observed in cap cells and ISCs (arrowheads). (D) Germarium of a female of genotype *P{ptc-GAL4} P{UAS-GFP}* (anti-GFP, green; anti-Hts, red). GFP is expressed in ISCs (arrowheads). (H) Distribution of EGFP-VAS fusion protein in flies carrying a *P{vas-egfp::vas}* transgene(anti-GFP, green; anti-Hts, red). Bracket in H indicates region 2 of the germarium. (I) Ovariole of female of genotype *P{vas-GAL4}*/+; *P{UAS.p-TkvAct} P{lacZ}Dad^{P1883}*/+ + (anti-GFP, green; anti-Hts, red). Note *lacZ* expression in germ cells at anterior tip of the germarium (top asterisk) and in egg chambers (bottom asterisks), but not in developing egg cysts in regions 2 and 3 of the germarium (bracket). (J) Summary of expression pattern of germline markers. Spec, spectrosome; Fus, fusome.

Epistatic analysis of *Mad^{12}* and *bam^{D86}*

To determine the approximate half lives of wild-type or *Mad* mutant GSCs, females of genotype *P{ry$^+$,hs-neo,FRT}40A/CyO*; *bam^{D86}/TM2,Ubx* (for wild-type GSCs) or *Mad^{12} P{ry$^+$,hs-neo,FRT}40A/CyO*; *bam^{D86}/TM2,Ubx* (for *Mad* mutant GSCs) were mated with males of genotype *P{hs-FLP}/Y; P{arm-lacZ} P{ry$^+$,hs-neo,FRT}40A*; *bam^{D86}/TM2,Ubx* in bottles and transferred daily. For both sets of crosses, the F1 progeny from the cross were heat-shocked twice at 37°C for 1 hour separated by 8-12 hours at stage P4 pre-pupae or P5 pupa, and ovaries from phenotypically Ubx F1 females were examined. Clones of wild-type GSCs were present in 38% of ovarioles (n=138) in 5-day-old females and 48% of ovarioles in 10-day-old females (n=79). GSCs mutant for *Mad^{12}* were present in 20% of ovarioles (n=73) from 5-day-old females, but only 4% of ovarioles (n=140) from 10-day-old females. Germline clones of wild-type or *Mad* cells in a *bam* background were examined in ovaries of non-Ubx F1 females from the above crosses.

Results

Dpp signaling in the germline is limited to GSCs and CBs

Cell fates within the germarium of the *Drosophila* ovariole can be characterized by a combination of morphological and molecular markers, which facilitate analysis of cell fate choice (Fig. 1A,J). One such marker, the Nos protein, is present in the GSCs and CBs, absent from 2-8 cell cysts, but is expressed at

high levels in 16 cell cysts (Verrotti and Wharton, 2000) (Fig. 1B). Other markers for cell type include Bam and the *Drosophila* CPEB homolog Orb, which is first expressed at high levels between the 8 and 16 cell cyst stage and ultimately becomes restricted to the presumptive oocyte (Lantz et al., 1994). Thus, a combination of molecular and morphological markers allows identification of all cell types within the germarium (Fig. 1J).

Because Dpp is both necessary for GSC maintenance and can be sufficient to cause overproliferation of cells with morphologies similar to GSCs, we wished to determine which germ cells within the germarium are responsive to Dpp signaling. To do so, we examined the spatial expression of *lacZ* driven by a P-element enhancer trap inserted near the Dpp target gene, *Dad*, which encodes an inhibitory Smad that in other developmental contexts has been shown to be transcriptionally activated by Dpp signaling (Tsuneizumi et al., 1997). Previous analysis (Xie and Spradling, 1998) had shown that GSCs lacking *Dad* have a longer half-life than do wild-type GSC clones, indicating that *Dad* functions within the germline. We found that *lacZ* is expressed only in spectrosome-containing cells and is absent from all fusome-containing cells (Fig. 1C). Moreover, putative GSC cells at the anterior tip of the germarium have an elevated level of *lacZ* expression compared with putative CB cells removed from the anterior tip (Fig. 1C,E,F). Using the criteria of cell position and level of *lacZ* expression, we found that wild-type ovarioles have an average of 2.3±0.9 putative GSCs, and 1.2±0.8 putative CBs (n=24). Moreover, *Dad-lacZ* is not expressed within the developing cysts and egg-chambers (Fig. 1G). Thus, within the germline, Dpp signaling is strictly limited to GSCs and CBs, and CBs appear to be less responsive to Dpp than their GSC sisters. Similar results were also reported recently by Kai and Spradling (Kai and Spradling, 2003).

The pattern of *Dad-lacZ* expression also allowed us to assay which somatic cells within the germarium are responsive to endogenous Dpp signaling. Although *lacZ* expression in somatic cells varied between individual preparations, in a significant fraction of wild-type ovarioles *Dad-lacZ* expression was visible in CpCs, but also in multiple somatic cells located in the same positions within regions 1 and 2A of the germarium as ISCs that express *patched* (Forbes et al., 1996) (Fig. 1D). This observation indicates that the CpCs and the ISCs are both exposed to Dpp and responsive to Dpp signaling, suggesting that Dpp is not limited to the GSC niche.

We then expressed a constitutively active form of the Dpp receptor Thickveins (TkvAct) in germ cells using a Gal4-UAS system optimized for germline expression (Rørth, 1998). When the *P{UAS.p-TkvAct}* construct was placed initially under the control of a *P{vas-GAL4}* driver, no overt change in morphological phenotype was observed. We assayed these ovarioles for Dpp signaling, as evidenced by *Dad-lacZ* expression. While the *vasa* promoter drives expression throughout the germline (Fig. 1H), we observed that the expression of the *Dad-lacZ* reporter gene in females of genotype *P{vas-GAL4}*/+; *P{UAS.p-TkvAct} P{lacZ}Dad*^{P1883}/ + + was not uniform. Specifically, in all ovarioles examined (n=15), *lacZ* was expressed in the GSCs and CBs and in the developing egg chambers (asterisks, Fig. 1I), but was absent from the developing cysts (bracket, Fig. 1I), suggesting that cyst cells are partially or completely refractory to Dpp signaling. Taken together, these data argue against a model in which the observed restriction of germline Dpp signaling to the GSC and CBs is caused only by the limited exposure of GSCs to Dpp ligand. Rather, these data strongly suggest that Dpp is present throughout the anterior germarium and that cell-intrinsic mechanisms operating within the developing cysts play an active role in downregulating Dpp signaling.

Constitutive Dpp signaling within the germline prevents GSC differentiation

Expression of the *P{UAS.p-TkvAct)* construct at higher levels, through use of a *P{Gal4::VP16-nos.UTR}* driver, resulted in production of 'tumorous' ovarioles that did not contain any differentiating egg chambers (Fig. 2A), but were filled with cells with all characteristics of wild-type GSCs. Specifically, all germline cells in these tumorous ovarioles contained spectrosomes (Fig. 2B,C), expressed *Dad-lacZ* (Fig. 2B), and stained for Nos-Myc (Fig. 3D). Moreover, no germline cells in any mutant ovariole (n=40) stained with the CB marker, Bam-C (Fig. 2C), nor expressed *bam* mRNA (not shown), as opposed to sibling wild-type ovaries in which Bam-C was expressed in CBs and young cysts (not shown). Many germ cells within these tumors are capable of undergoing an apparent self-renewal division, as evidenced by staining with an anti-Histone H3 antibody that recognizes cells in M phase (not shown), and, as described below, are capable of differentiation. Thus, using the spectrum of markers available to us, we conclude that cell autonomous activation of Dpp

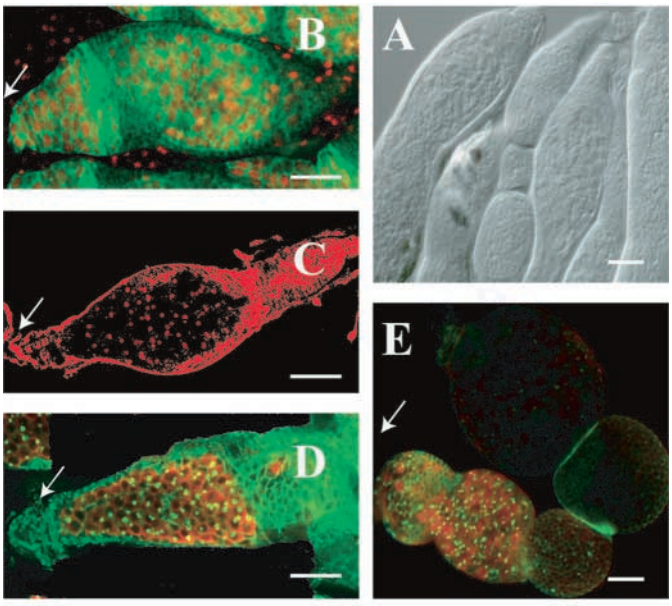


Fig. 2. Characterization of ovarioles from females expressing the TkvAct receptor. All females were of genotype *P{UAS.p-TkvAct}*/*P{Gal4::VP16-nos.UTR}*. (A) Nomarski image of multiple tumorous ovarioles. (B) Confocal projection of tumorous ovariole from 2-day-old female carrying the *Dad-lacZ* transgene, indicating all germ cells express Tkv and *Dad-lacZ* (anti-Tkv, green; anti-*lacZ*, red). (C) Tumorous ovariole from a 1-day-old female indicating lack of BamC expression (anti-BamC, green; anti-spectrin, red). (D,E) Tumorous ovarioles from 3-day-old (D) and 1-week-old (E) females (anti-Hts, green; anti-Vasa, red). Arrow indicates position of terminal filament. Scale bars: 10 μm.

signaling in the germline produces cells with morphological, molecular and functional characteristics indistinguishable from wild-type GSCs.

Expression of the TkvAct receptor in the germline, however, is not sufficient to maintain putative GSC identity indefinitely. Whereas all germ cells in tumors from young females (Fig. 2D) express the germline-specific RNA helicase Vasa (Lasko and Ashburner, 1988), germ cells in the posterior of tumorous ovarioles of females greater than 5 days old fail to express Vasa (Fig. 2E) and show decreased Nos-Myc expression (not shown). Moreover, some germ cells undergo apoptosis (not shown). We note that germ cells in aged *bam* mutant females display identical phenotypes (not shown), thus we infer this phenotype is not due to prolonged Dpp signaling, but may result from any of a number of causes, including hypoxia, loss of niche signals or inability to undergo differentiation.

These data demonstrate that Dpp has no additional, obligate role in somatic cells for maintenance of GSC-like identity, and that Dpp signaling initially can maintain GSC-like cells independent of the somatic niche. However, as the majority of these germ cells are not in contact with the somatic niche, and thus may not receive contact-dependent niche signals, these germ cells could represent an intermediate state between a GSC and a CB. A similar hypothesis was proposed recently by Gilboa et al. (Gilboa et al., 2003). For simplicity of nomenclature, though, we will refer to these cells in the remainder of this paper as GSCs, but we recognize that a more extensive panel of molecular markers will be required to determine whether these cells are in fact identical to wild-type GSCs.

Bam expression promotes GSC differentiation in part by downregulation of Dpp signaling

We then wished to investigate whether Bam function would be sufficient to promote GSC differentiation in the presence of constitutive Dpp signaling. To do so, we heat shocked flies of genotype *P{hs-bam.O}/ +; P{UAS.p-TkvAct}/ P{Gal4::VP16-nos.UTR}* daily starting at 7 days of development and observed their ovaries at various periods after eclosion. Our data indicate that expression of Bam is sufficient to completely overcome the effects of constitutive Dpp signaling to promote normal germline differentiation.

Examination of ovarioles 3 or 4 days after eclosion revealed significant rescue of the tumorous ovariole phenotype (Fig. 3A), with all ovarioles containing egg chambers with large, polyploid nuclei and germline expression of Orb. Although many of these egg chambers had a normal 15:1 nurse cell to oocyte ratio, some contained an abnormal number of germ cells (Fig. 3A and inset). Extension of the heat shock to 7 days post-eclosion and assay of the ovarian phenotype 3 days later resulted in the production of ovarioles with completely normal morphology (Fig. 3B). The germaria of many rescued ovarioles had a wild-type appearance with a small number of spectrosome-containing cells in the GSC niche (Fig. 3C). However, some germarium were devoid of germ cells, suggesting that germ cells in the GSC niche may be more, but not completely, resistant to the effects of Bam. Many females subject to this heat shock regiment laid eggs with normal follicular morphology; however, the eggs did not differentiate cuticle, possibly because of the dorsalizing effects of the TkvAct receptor on embryonic pattern.

We then determined whether ubiquitous ectopic expression of Bam in tumorous ovarioles caused the same pattern of changes in morphology and gene expression during initial GSC differentiation as was observed in wild-type ovarioles. Flies of genotype *P{hs-bam.O}/+; P{nos-myc.V}/+; P{UAS.p-TkvAct} P{lacZ}Dad^{P1883}/P{GAL4::VP16-nos.UTR}* were heat shocked daily starting at 9 or 10 days of development and their ovaries were examined 2 days after eclosion for the expression of the Nos-Myc and *Dad-lacZ* markers. Although Nos was present in all germ cells in control tumorous ovarioles not subject to heat shock (Fig. 3D), Nos displayed a dynamic pattern of expression in heat-shocked ovarioles that was identical to its pattern in the wild type (Fig. 3E). Specifically, Nos was expressed in all spectrosome-containing cells in the heat-shocked ovarioles (100%, *n*=87 cells), was absent in two- to eight-cell cysts (18%, *n*=17 cysts), and became upregulated in all 16-cell cysts (100%, *n*=15 cysts). These results demonstrate that the dynamic pattern of wild-type Nos expression is recapitulated after ectopic Bam expression, raising the possibility that Nos downregulation is necessary for cyst formation.

We were particularly interested in examining the pattern of *Dad-lacZ* expression in these heat-shocked tumorous ovarioles. If the spatial extent of Dpp signaling in wild-type germaria were controlled solely by ligand availability, then we would expect to see that germ cells in the heat-shocked ovarioles would continue to express *Dad-lacZ*. Alternatively, if Dpp signaling is actively downregulated during germ cell differentiation, then differentiating germ cells in the heat shocked ovarioles should not express *Dad-lacZ*.

Comparison of confocal projections of heat-shocked ovarioles to control, non heat-shocked ovarioles that were processed identically revealed a decrease in the amount of *lacZ* present in the germ cells of the heat-shocked ovarioles compared with the controls (78%, *n*=41 ovarioles). Significant downregulation (compare Fig. 3F to Fig. 3G) but not elimination (Fig. 3H) of *Dad-lacZ* levels occurred prior to downregulation of Nos during cyst formation and was specific to the germline, as *Dad-lacZ* expression remained at high levels in somatic cells (Fig. 3G). We correlated the expression of *Dad-lacZ* in these heat shocked ovarioles with two distinct markers for the state of germline differentiation, the presence of spectrosomes or fusomes in a given germ cell, and whether any surrounding germ cells expressed Orb. Strikingly, *lacZ* was never present in germ cells undergoing overt differentiation, either those that had fusomes (Fig. 3I; 0%, *n*=20 cysts), or expressed Orb (not shown). Moreover, the decrease in *lacZ* expression is apparent (67%, *n*=168 cells) in many spectrosome-containing cells (compare Fig. 3J with 3K). In general, downregulation of *lacZ* expression in spectrosome-containing cells was more evident in ovarioles with signs of overt differentiation, such as cyst formation. The downregulation of *lacZ* expression is not likely to be due to action of Bam on either the UAS-GAL4 system or the stability of *lacZ*, as flies of genotype *P{hs-bam.O}/P{UAS.p-lacZ}; P{GAL4::VP16-nos.UTR} bam^{D86}/bam^{D86}*, in which the same regulatory constructs were used to drive *lacZ* expression, displayed *lacZ* staining throughout the germline prior to (Fig. 3L), and after a heat shock expression of Bam that caused germline cyst production (Fig. 3M). Taken together, these data indicate that expression of Bam leads to a block in Dpp

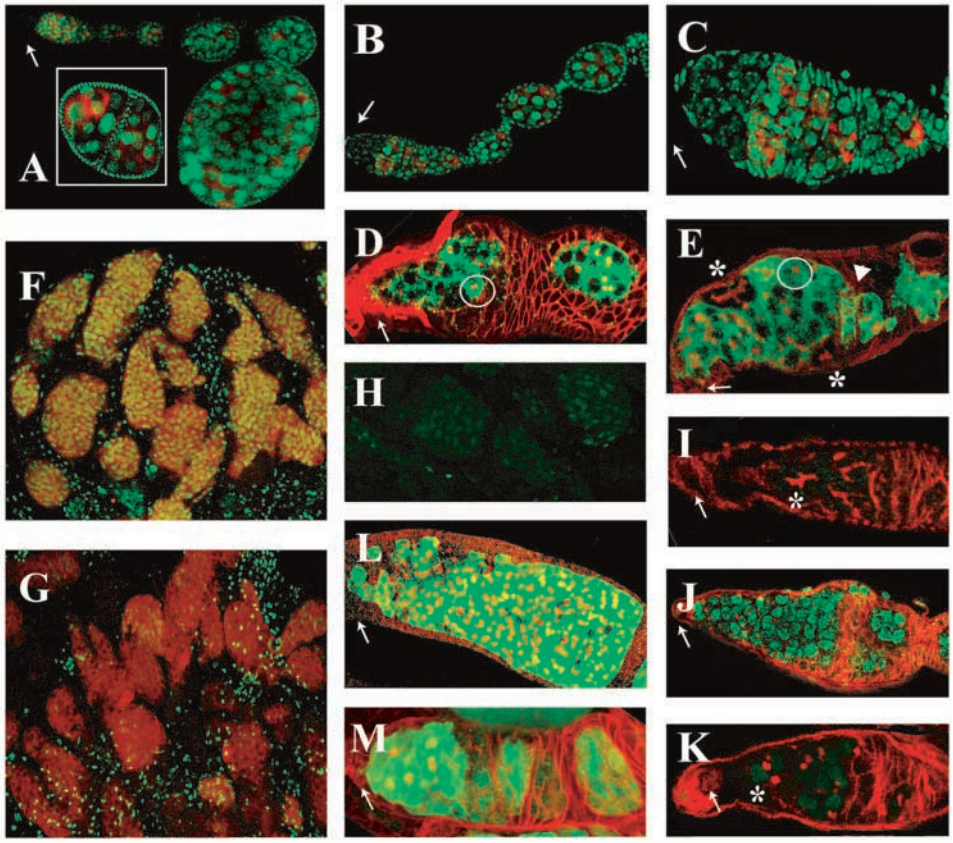


Fig. 3. Bam promotes differentiation of TkvAct ovarioles in part by downregulating Dpp signaling prior to overt GSC differentiation. (A-C) Fluorescence (A) and confocal (A inset, B,C) projections of ovarioles from females of genotype *P{hs-bam.O}/ +; P{UAS.p-TkvAct)/ P{Gal4::VP16-nos.UTR}*. Anti-Orb (red). (A) DAPI staining (green). Ovariole of female subject to daily heat shock from day 10 of development. (Inset) Abnormal egg chamber from an ovariole of a similarly treated female. (B,C) Yopro-1 staining (green) of (B) ovarioles and (C) germarium of 10-day-old female subject to daily heat-shock from day 9-10 of development until 7 days post-eclosion. Note complete rescue of the tumorous phenotype (B), with differentiation of wild-type appearing egg chambers with 15 polyploid nurse cells and localization of Orb to the presumptive oocyte, and wild-type appearance of the germarium (C). (D-K) Confocal projections (D-G) or confocal sections (H-K) of germaria or ovarioles from females of genotype *P{hs-bam.O}/ +; P{nos-myc.V}/ +; P{UAS.p-TkvAct} P{lacZ}Dad^{P1883}/ P{GAL4::VP16-nos.UTR}*. (D,E) Anti-spectrin (red), anti-Myc (green). In a non-heat shocked control germarium (D), all germ cells express Nos (circle, one example). In a germarium from a 2-day-old adult female heat shocked daily from day 9-10 of development (E), all spectrosome-containing cells express Nos (circle, one example). Nos is downregulated in all germline cysts that have between two and eight cells (asterisks), but is re-expressed at high levels in 16 cell cysts (arrowhead). (F,G) Anti-Myc (red), anti-*lacZ* (green). Ovarioles from 2-day-old female without heat shock (F), or with daily heat shocks starting at day 10 of development (G). In heat-shocked ovarioles, the level of Nos-Myc is unchanged in germ cells, while the level of *Dad-lacZ* expression is significantly reduced in germ cells, but not in follicle cells. (H) Anti-*lacZ* (green). A confocal section from part of the projection in G, showing *lacZ* expression is reduced but not eliminated in the germline. (I-K) Germaria from control (J) and heat shocked (I,K) females (anti-*lacZ*, green; anti-Hts, red). Compared with control females (J), heat-shock causes reduction, but not elimination of *lacZ* expression in spectrosome containing cells (K, asterisk), and complete elimination of *lacZ* expression in fusome-containing cysts (I, asterisk). (L,M) Confocal projections of ovarioles from females of genotype *P{UAS-lacZ.p}/ P{hs-bam.O}; bam^{D86}/ P{GAL4::VP16-nos.UTR} bam^{D86}* (anti-Hts, red; anti-*lacZ*, green). Germarium of non-heat shocked control (L) and germarium of 2-day-old female heat shocked daily from day 9-10 of development (M), showing rescue of tumorous phenotype as evidenced by differentiating egg chambers. Levels of *lacZ* are not reduced after heat shock. (A-E,I-M) Arrow indicates terminal filament.

signaling downstream of Tkv activation, and that this block occurs prior to onset of overt GSC differentiation.

Bam plays an instructive role in germline differentiation

Previous experiments (Xie and Spradling, 1998) have shown that loss of Dpp signaling in the germline leads to failure to maintain a GSC fate. Conversely, Bam has been shown to be both necessary and sufficient to promote CB differentiation (McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). The data presented above raise the possibility, however, that Bam could promote CB differentiation solely by blocking Dpp signaling. To determine whether Bam has additional functions during CB differentiation, we used the FLP-FRT system (Xu and Rubin, 1993) to make clones of germ cells doubly mutant for *bam* and the Dpp signal transducer *Mad*.

Previous work (Xie and Spradling, 1998) indicated that GSCs homozygous for *Mad*^{12} are not maintained over time. We repeated these experiments and arrived at similar conclusions (Materials and methods). Although the fates of GSCs lacking *Mad* function were not ascertained in these experiments, in both our analysis and that of Xie and Spradling (Xie and Spradling, 1998), young *Mad* mutant cysts were found in the anterior germarium a significant time after induction of mitotic recombination, suggesting they were progeny of mutant GSCs that had undergone differentiation. Moreover, we found that all such cysts (n=23) were phenotypically normal, indicating Dpp signaling is not required for cyst differentiation.

We were unable to obtain either wild-type or *Mad* mutant germ cell clones by inducing mitotic recombination in adult *bam* females, suggestive of a very slow rate of germ cell division in the adult. However, small clones of germ cells doubly mutant for *Mad* and *bam*, containing on average one or two cells, could be obtained after induction of recombination in pupal stages. These doubly mutant cells contained round spectrosomes and were identical in morphology to *bam* single

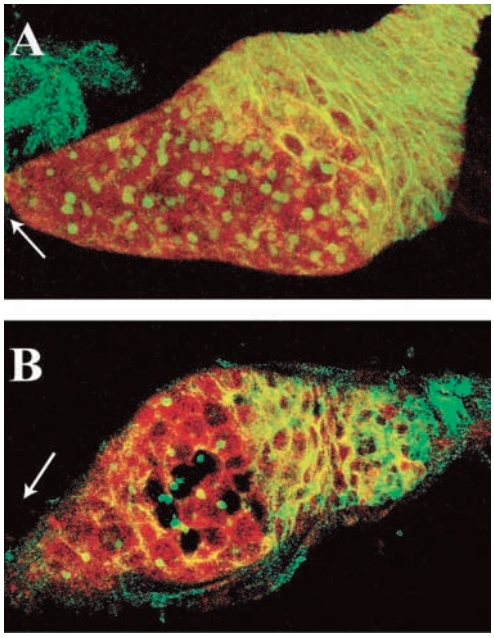


Fig. 4. *bam* is epistatic to *Mad*. Confocal projections of tumorous ovarioles from young (under 5-day-old) females of genotype *P{hs-FLP};P{arm-lacZ} P{ry⁺,hs-neo,FRT}40A/ Mad¹² P{ry⁺,hs-neo,FRT}40A*; *bam^D86* (anti-Hts, green; anti-*lacZ*, red). (A) Non heat-shocked control. (B) After heat-shock-induced mitotic recombination, doubly mutant germ cells, which do not express *lacZ*, had rounded spectrosomes, indicative of failure to form cyst cells. Arrow indicates terminal filament.

mutant germ cells (100%, *n*=30 ovarioles, compare Fig. 4B with 4A), indicating these cells did not form germline cysts. Because Dpp signaling is not required for cyst differentiation, these data indicate that Bam plays an essential role in CB differentiation independent of its function in downregulating Dpp signaling.

Bam acts redundantly with Smurf to downregulate Dpp signaling in the germline

As ectopic expression of Bam was sufficient to downregulate Dpp signaling in the TkvAct ovarioles, we wished to determine whether the number of germ cells responsive to Dpp signaling was increased in *bam* mutant ovarioles. We found that *lacZ* was expressed in 2.2±0.6 (*n*=27) germ cells in the anterior tip of *bam* mutant germaria (Fig. 5A), which is equivalent to the number of cells with elevated *Dad-lacZ* expression in wild-type ovarioles. Similar data were also reported recently by Kai and Spradling (Kai and Spradling, 2003). From these data, we conclude that *bam* action is not absolutely required to downregulate Dpp signaling during GSC differentiation. Moreover, these data suggest *bam* mutant germ cells may represent a novel or intermediate step in the process of germline differentiation, as the great majority of *bam* mutant germ cells have a molecular signature different from either a GSC or a CB.

One resolution to the apparent paradox of the sufficiency of Bam, but not its necessity, for downregulation of Dpp signaling during GSC differentiation would be if Bam were functionally redundant with the action of a second gene. Previous work from our laboratory identified two classes of mutations that elevate Dpp signaling in the embryo (Podos et al., 2001) (S. Podos, Y.-C. Wang and E.L.F., unpublished). Loss-of-function mutations in the *Smurf* gene, which encodes an ubiquitin protein ligase, cause spatial expansion and temporal prolongation of Dpp signaling (Podos et al., 2001), probably through the failure to degrade active forms of Mad (Liang et al., 2003). An embryonic phenotype similar to that caused by *Smurf* mutations is also observed when certain dominant gain-of function mutations in the type I BMP receptor *saxophone* (*sax*) are placed in the background of three copies of the *dpp⁺* gene (S. Podos, Y.-C. Wang and E.L.F. unpublished), suggesting that the *sax* mutations also cause temporal prolongation of Dpp signaling, possibly because of failure to downregulate the receptor complex.

We then determined how these classes of mutations affected the spatial extent of Dpp signaling within the GSC niche. Ovarioles from *Smurf ^15C* mutant females had a significantly greater number (4.0±2.0, *P*<0.001, Neuman-Kuels test, *n*=20) of high-*lacZ* expressing cells than did wild-type ovarioles (Fig. 5C). Although the number of high-*lacZ* expressing cells in ovarioles of females carrying three copies of *dpp⁺* (2.7±1.3, *P*>0.05, *n*=18) and in ovarioles from females carrying the dominant *sax^B18* allele (3.2±1.3, *P*>0.05, *n*=9) was not significantly different from wild type, ovarioles from females carrying both the *dpp⁺* duplication and the *sax^B18* allele had a significantly greater number (4.0±1.7, *P*<0.001, *n*=25) of high-*lacZ*-expressing cells than did wild type (Fig. 5B). Thus, mutations that elevate or prolong Dpp signaling can increase the number of putative GSCs within the niche, suggesting mechanisms that control perdurance of Dpp signaling could also play a role in limiting Dpp signaling within the germline.

To determine whether either of these genotypes could synergize with *bam* mutations to result in a more extensive deregulation of Dpp signaling within the germline, we constructed *bam* mutant females that also carried the *Smurf* or *sax* mutations. We found that in many multiply mutant ovarioles the spatial extent of Dpp signaling was significantly expanded, so that *Dad-lacZ* expression was observed in germ cells throughout the entire ovariole. This phenotype was not uniform, however, and could vary even in ovarioles from a single female (e.g. Fig. 5D-F contains images from a single confocal section of ovarioles from one female). In general, ovarioles from females of these mutant genotypes fell into two phenotypic classes. The first class showed a substantial increase in the number (12.1±6.0, *n*=21 *sax^B18 Tp(2;2)DTD48*/ + +; *bam^D86* ovarioles; 11.1±3.8, *n*=17 *Smurf^15C*; *bam^D86* ovarioles) of germ cells expressing high levels of *lacZ*. In these ovarioles, *lacZ*-expressing germ cells were found associated with somatic cells and/or distributed in a salt-and-pepper fashion throughout the ovariole [Fig. 5D,E,G (*sax^B18 Tp(2;2)DTD48*/+ +; *bam^D86* ovarioles), upper ovariole in Fig. 5H,J (*Smurf^15C*; *bam^D86* ovarioles)]. Occasionally, we observed a *Smurf^15C*; *bam^D86* ovariole in which most, if not all, germ cells within the tumor expressed *lacZ* (ranging from 40 to 60 germ cells, *n*=2) (Fig. 5I). The second class of ovarioles showed no significant increase in the number (3.3±0.6, *n*=12 *sax^B18 Tp(2;2)DTD48*/+ +; *bam^D86* ovarioles; 3.0±1.2, *n*=12 *Smurf^15C*; *bam^D86* ovarioles) of germ cells expressing *Dad-lacZ*, and the *lacZ*-expressing cells were confined to the anterior region of the tumor close to the terminal filament (Fig.

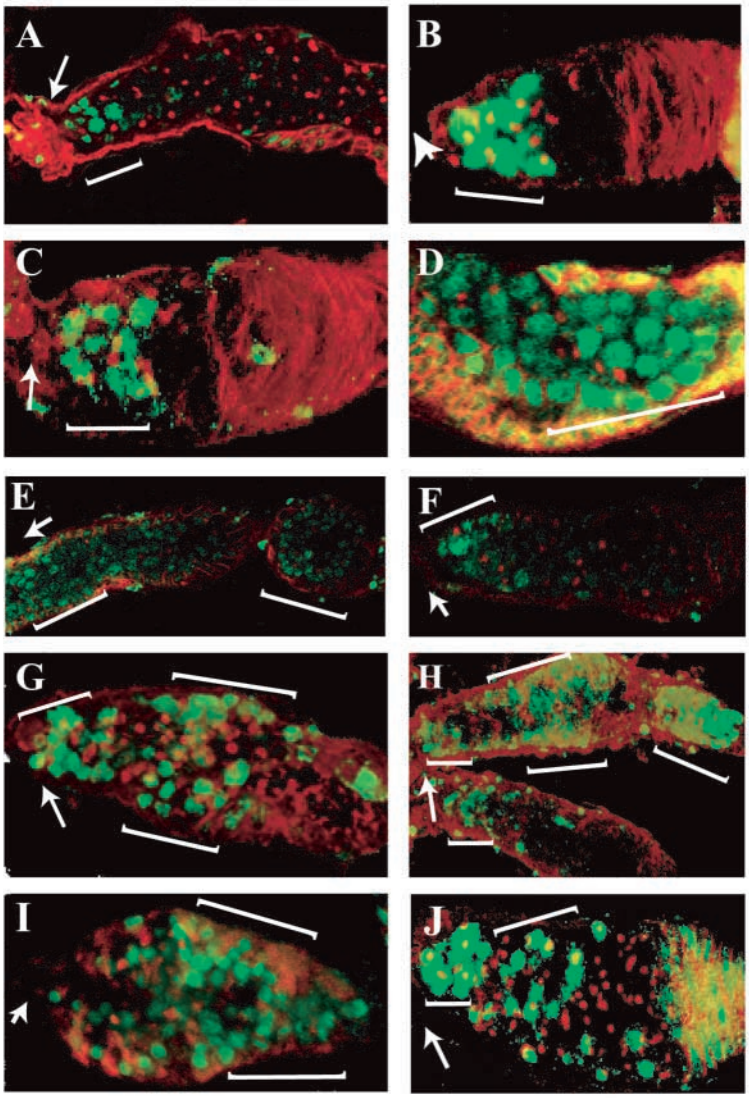


Fig. 5. Bam acts redundantly with Dpp signaling components to downregulate Dpp signal transduction (anti-*lacZ*, green; anti-Hts, red). (A-C,G-J) Confocal projections of ovarioles from one- to two-day-old females; (D-F) three images from a single confocal section of a female of the same age. (A) A germarium of an ovariole from a bam^{D86} $P\{lacZ\}Dad^{P1883}/bam^{D86}$ female showing that *Dad-lacZ* expression is confined to germ cells in the anterior tip of the germarium. (B) A germarium of ovariole from sax^{B18} *Tp(2;2)DTD48*/+ +; $P\{lacZ\}Dad^{P1883}$/+ female. (C) A germarium of ovariole from $Smurf^{15C}$; bam^{D86} $P\{lacZ\}Dad^{P1883}$/+ female. (D-G) Ovarioles from females of genotype sax^{B18} *Tp(2;2)DTD48*/+ +; bam^{D86} $P\{lacZ\}Dad^{P1883}/bam^{D86}$. (D-F) Three images from a single confocal section, illustrating the variability of the extent and intensity of *Dad-lacZ* expression: (D) posterior part of the ovariole with at least 20 germ cells that have high-level *Dad-lacZ* expression; (E) ovariole in which many germ cells express intermediate levels of *Dad-lacZ*; (F) ovariole with *Dad-lacZ* expression only in germ cells at anterior tip. (G) Expression of *Dad-lacZ* in germ cells scattered in salt-and-pepper fashion throughout ovariole. (H-J) Ovarioles from $Smurf^{15C}$; bam^{D86} $P\{lacZ\}Dad^{P1883}/bam^{D86}$ females. (H) Upper ovariole, expression of *Dad-lacZ* throughout ovariole in germ cells adjacent to somatic cells. Lower ovariole, expression of *Dad-lacZ* in approximately five cells at anterior tip of germarium. (I) An ovariole containing at least 40 *Dad-lacZ* positive germ cells. (J) Expression of *Dad-lacZ* throughout ovariole in a salt-and-pepper fashion. In all panels, arrow indicates anterior tip of the germarium, brackets indicate germ cells expressing *Dad-lacZ*.

5F, a sax^{B18} *Tp(2;2)DTD48*/+ +; bam^{D86} ovariole; Fig. 5H lower ovariole, a $Smurf^{15C}$; bam^{D86} ovariole). Our data thus indicate that in both double mutants, unlike any of the single mutants, germ cells throughout the ovariole can be responsive to Dpp signaling. We conclude that during wild-type development Bam downregulates Dpp signaling during germline differentiation, but that its activity is functionally redundant with certain components of the Dpp signaling pathway.

Discussion

The prevalent model for Dpp action within the ovary is that it is a local niche signal whose activity is permissive for GSC maintenance. In this model, only GSCs within the niche are exposed to Dpp protein and removal of the CB from the niche lessens or eliminates exposure to the ligand. Moreover, the only postulated function of Dpp is to repress the transcription of *bam* within the GSCs. The data presented in this paper reveal additional aspects of Dpp function in GSC maintenance. Our results strongly suggest that Dpp ligand is not restricted to the niche but rather is present throughout the anterior germarium. We present data that the observed specificity of Dpp signaling to the GSCs and CBs is due to functionally redundant mechanisms that operate in the germline to actively downregulate Dpp signaling during GSC differentiation. One of these mechanisms is Bam itself, thus establishing a negative feedback loop between the actions of the two genes. Our findings indicate GSC differentiation is correlated with downregulation of Dpp signaling, raising the possibility that Dpp signaling plays an active role in GSC maintenance, and that GSC differentiation requires both the presence of Bam and the absence of Dpp signaling.

Model for control of Dpp signaling within the germline

If GSCs and CBs are exposed to equivalent amounts of Dpp protein, as is suggested by both the transcription pattern of the Dpp gene (Xie and Spradling, 2000) and the expression of *Dad-lacZ* in the CpCs of the niche and the ISCs posterior to the niche, then it is likely that the observed reduction in *Dad-lacZ* expression between the GSC and the CB results from intracellular modulation of the strength of the Dpp signal. One hallmark of the GSC is its invariant plane of division. We propose that the differential Dpp signaling between the GSC and CB sign results from an intracellular modulation of Dpp signal strength between the two daughter cells, either by the asymmetric segregation of one or more cellular components that modulate Dpp signaling, or by loss of a contact-based niche signal that elevates Dpp signaling preferentially within the GSCs. Removal of the CB cell from the niche thus results

in partial downregulation of Dpp signaling. A lower level of Dpp signaling in the CB cell results in the transcription of Bam, which plays multiple roles in CB differentiation, one of which is to cause the daughters of the CB cell to become refractory to further Dpp signaling. Thus, sequential regulatory mechanisms cooperate to ensure an irreversible change in the fate of the GSC cell within two generations (Fig. 6).

Smurf and *sax* mutations prolong Dpp signaling in the niche

Loss-of-function mutations in *Smurf* and gain-of-function mutations in *sax* increase the number of GSCs, suggesting they may perturb the proposed intracellular modulation of Dpp signaling that occurs between the GSC and CB. However, these data are not sufficient to determine whether this proposed modulatory pathway acts through direct regulation of the functions of one or both of these gene products, or whether the proposed pathway acts in parallel to these genes. In the embryo, loss of *Smurf* activity results in a ligand-dependent elevation of Dpp signaling that has greater, but not indefinite, perdurance (Podos et al., 2001), suggesting that Dpp signaling in *Smurf* mutants, and by inference *sax* mutants, is still responsive both to the amount of ligand and to the presence of other negative regulatory mechanisms. In the ovary, the *Dad-lacZ*-expressing germ cells in the *Smurf* and *sax* mutants (Fig. 5B,C) fill the region of the anterior germarium that roughly corresponds to the spatial extent of *Dad-lacZ* expression in the somatic cells of region 1 and 2A of a wild-type germarium (Fig. 1C,D), suggesting that potentially all germ cells in region 1 and 2A of the *Smurf* and *sax* germaria are equally and fully responsive to the Dpp ligand. We propose that GSCs in the *Smurf* and *sax* germaria ultimately undergo normal differentiation because in the more posterior regions of the germaria the amount of Dpp ligand may be reduced to a level that allows *bam* transcription, which further reduces Dpp signaling and causes cyst differentiation.

Bam downregulates Dpp signaling downstream of receptor activation

The reduction in Dpp signaling between the GSC and the CB releases Bam from Dpp-dependent transcriptional repression (Chen and McKearin, 2003b), and we have shown that one, but not the only, function of Bam is to downregulate Dpp signaling downstream of receptor activation prior to overt GSC differentiation. This is the first molecular action ascribed to Bam, and these data could provide an entry point to elucidate the biochemical basis of the function of Bam in CB differentiation. Further work will be necessary to determine whether the action of Bam on the Dpp pathway is direct or indirect, whether Bam action results in the reduction or complete elimination of Dpp signaling in the developing cysts, and which step in the intracellular Dpp signal transduction pathway or expression of Dpp target genes is affected by Bam action. However, it is possible that initial insights into Bam function can be made by comparing the thresholds for Dpp signaling readouts in the developing wing disc of the larva to the data we and others have obtained in the germarium. In the wing disc, Dpp diffuses from a limited source to form a gradient throughout the disc that displays different thresholds for multiple signaling readouts. Specifically, *Dad-lacZ* is transcribed in response to high and intermediate levels of Dpp,

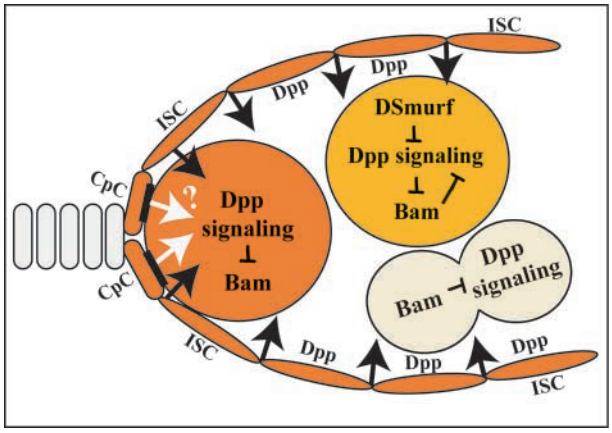


Fig. 6. Model for the regulation of Dpp signaling in the GSC and CBs. See text for details.

but does not respond to the lowest levels of ligand (Minami et al., 1999). An antibody exists that recognizes the active phosphorylated form of Mad, pMad (Persson et al., 1998). In the wing disc, high level staining with the pMad antibody is present in only a subset of cells that express high levels of *Dad-lacZ*, suggesting that in this tissue the pMad antibody is less sensitive to Dpp signaling than is *Dad-lacZ* expression (Minami et al., 1999; Tanimoto et al., 2000; Teleman and Cohen, 2000). Intriguingly, Gilboa et al. (Gilboa et al., 2003) recently reported that in the ovariole pMad staining is visible in the GSCs, CBs and the developing cysts. Because we never observe *Dad-lacZ* expression in the developing cysts, these results could suggest that the relative sensitivities of these two reagents are reversed within the germline. Alternatively, if the reagents have the same relative sensitivities in the two tissues, the data suggest that Bam could act, probably at a post-transcriptional level, to downregulate Dpp signaling downstream of Mad activation.

Functional redundancy in control of Dpp signaling in the germ line

We have shown that the pattern of *Dad-lacZ* expression observed in the *Smurf; bam* and *sax*; *bam* double mutant ovarioles is qualitatively different from that observed in any of the single mutant ovarioles. Although *Dad-lacZ* expression is only observed at the anterior tip of the germarium of each single mutant, many, but not all, of the double mutant ovarioles contain germ cells throughout the ovariole that express high levels of *Dad-lacZ*. From these data, we conclude that two redundant pathways downregulate Dpp signaling in the germline, and that in the single mutants, the action of the remaining active pathway is sufficient to constrain Dpp responsiveness to the anterior tip of the germarium. However, we note that not all doubly mutant ovarioles display a spatial expansion of Dpp signaling, and that this variability can even be observed in ovarioles from a single female. We propose that the observed variability results because the *Smurf* and *sax* mutations have modulatory effects on Dpp signaling that are both dependent on the presence of ligand and are sensitive to additional mechanisms that downregulate Dpp signaling. In both the *Smurf*; *bam* and *sax*; *bam* ovarioles, the germ cells that

express *Dad-lacZ* are observed throughout the ovariole, but are more likely to be near somatic cells. It is possible that the variability in *Dad-lacZ* expression occurs because of a non-uniform distribution of the Dpp ligand. Nevertheless, there is not a consistent correlation between the domains of *Dad-lacZ* expression in the somatic and germ cells, suggesting that there may be additional germline intrinsic factors that affect Dpp signaling.

We thank Steven Cohen, Anthony Mahowald, Dennis McKearin and Allan Spradling for helpful discussions and reagents. We thank Daniel Cox, Ruth Lehmann, Pernille Rørth and the University of Iowa for reagents; and Saturo Kobayashi, Anthony Mahowald, Tetsuya Tabata, Robin Wharton, the Bloomington *Drosophila* Stock center and the BDGP for fly stocks. We thank Chris Li for statistical analysis, Kirsten Hanson and Donald Vander Griend for plasmid constructions, and Monica Rohrschneider and Mihoko Kato for help with the heat shock experiments. We thank Yu-Chiun Wang and Barbara Page for discussions, Ben Glick for support with microscope imaging, and Victoria Prince and Urs Schmidt-Ott for comments on the manuscript. This work was supported by the Fletcher Scholar Award from the Cancer Research Foundation, by the Faculty Research Fund of the University of Chicago and by a grant from the National Institutes of Health (GM50838).

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